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2. Patent application number *(The Patent Office will fill in this part)* — 9905310.0 -9 MAR 1999

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

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APPLICATION FILED 19.05.00.

Patents ADP number *(if you know it)* — 7409436005

If the applicant is a corporate body, give the country/state of its incorporation — United Kingdom

4. Title of the invention — THERAPY AND USE OF COMPOUNDS IN THERAPY (4)

5. Name of your agent *(if you have one)*

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11. I/We request the grant of a patent on the basis of this application.

Signature: Eric Potter Clarkson
ERIC POTTER CLARKSON

Date: 9 March 1999

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THERAPY AND USE OF COMPOUNDS IN THERAPY (4)

The present invention relates to therapy and the use of compounds in therapy. In particular, it relates to the treatment and prevention of endotoxin-mediated immune activation in acute and chronic heart failure (CHF).

Chronic heart failure is a heterogeneous syndrome with an overall adverse prognosis. It is a disease in which there is a failure to pump enough bloodaround the body to meet its needs. Two particular predictors of adverse prognosis are neurohormonal abnormalities (Packer (1992) *J Am Coll Cardiol* **20**, 248-254) and the development of cachexia (Abel *et al* (1976) *Arch Surg* **111**, 45-50).

The syndrome of cardiac cachexia has been recognized for many centuries (Katz *et al* (1962) *Br Heart J* **24**, 257-264), but little is known about the mechanisms of the transition from heart failure to cardiac cachexia. Even the definition of cachexia and the characteristics of the cachectic patient are controversial. More than 30 years ago, the pathogenesis of cardiac cachexia was linked to dietary and metabolic factors (Pittman & Cohen (1964) *New Eng J Med* **271**, 403-409). In 1990, Levine *et al* ((1990) *New Eng J Med* **323**, 236-241) and subsequently others (McMurray *et al* (1991) *Br Heart J* **66**, 356-358; Dutka *et al* (1993) *Br Heart J* **70**, 141-143) showed the TNF-α in plasma is increased in patients with severe heart failure and coexisting cardiac cachexia, as in other wasting disorders. The plasma concentrations of TNF-α partly reflect the local tissue concentration, which is more closely related to muscle wasting (Tracey *et*

al (1990) *J Clin Invest* **86**, 2014-2024). Cytokine activation is a potential causal mechanism for the development of cachexia.

Cardiac cachectic patients suffer from loss of both muscle (ie protein reserves) and fat tissue (ie energy reserves), indicative of increased catabolism. An increased resting metabolic rate, regulated primarily by thyroid hormones (Himms-Hagen *et al* (1993) In: Grandier R. Stock, eds, Mammalian Thermogenesis, Chapman & Hall, London, UK) and catecholamines (Poehlman & Danforth (1991) *Am J Physiol* **261**, E233-E239), has been reported in CHF patients (Poehlman *et al* (1994) *Ann Intern Med* **121**, 860-862). Cortisol, another catabolic hormone, is also increased in untreated severe congested heart failure patients (Anand *et al* (1989) *Circulation* **80**, 299-305). Less is known about anabolic metabolism in heart failure. Anand *et al* ((1989) *Circulation* **80**, 299-305) found hGH to be greatly increased (≈10-fold) in untreated patients with severe heart failure. To date, these results have not been confirmed by others. Increased plasma insulin levels and insulin resistance occur in patients with CHF (Swan *et al* (1994) *Eur Heart J* **15**, 1528-1532).

The neurohormonal hypothesis (Packer (1992) *J Am Coll Cardiol* **20**, 248-254) postulates that heart failure progresses because activated endogenous neurohormonal systems exert a deleterious effect on the heart and circulation. Several studies have found neurohormonal activation to be strongly related to mortality (Cohn *et al* (1984) *New Eng J Med* **311**, 819-823; Swedberg *et al* (1990) *Circulation* **82**, 1730-1736; Francis *et al* (1993) *Circulation* **87**, (Suppl VI) VI-40 - VI-48) but different hormones correlate only weakly with each other (Swedberg *et al* (1990) *Circulation* **82**, 1730-1736). Norepinephrine and plasma renin activity were found not

to be related to peak oxygen consumption (peak VO_2) or LVEF (Francis *et al* (1993) *Circulation* **87**, (Suppl VI) VI-40-VI-48). Left ventricular function, exercise capacity, clinical status, and sympathetic activation were independently related to the progression of CHF (Francis *et al* (1993) *Circulation* **87**, (Suppl VI) VI-40-VI-48).

Anker *et al* (1997) *Circulation* **96**, 526-534 describes a study of the hormonal changes and catabolic/anabolic imbalance in CHF and concludes that cachexia is more closely associated with hormonal changes in CHF than conventional measures of the severity of CHF and suggests that the syndrome of heart failure progresses to cardiac cachexia if the normal metabolic balance between catabolism and anabolism is altered.

Anker *et al* (1997) *The Lancet* **349**, 1050-1053 suggests that the cachectic state is a strong independent risk factor for mortality in patients with CHF.

Anker *et al* (1997) *J Am Coll Cardiol* **30**, 997-1001 describes investigations of tumour necrosis factor (TNF) and steroid metabolism is CHF and concludes that there is an increase in TNF and its soluble receptor in CHF and that this increase is associated with a rise in the cortisol/DHEA (catabolic/anabolic) ratio. These changes correlate with body mass index and clinical severity of heart failure, suggesting a possible etiological link.

Anker *et al* (1997) *Am J Cardiol* **79**, 1426-1430 suggests that a chronic endotoxin challenge may cause immune activation in CHF and indicates that patients with high soluble CD14 levels have markedly increased levels

of TNF-α, soluble TNF receptors 1 and 2, and intracellular adhesion molecule-1.

Starr *et al* (1995) Direct action of endotoxin on cardiac muscle *Shock* 3(5), 380-384 suggest that endotoxin directly affects the contractile response of cardiac muscle to calcium.

Endotoxin is known to be the strongest biological stimulus for cytokine production, in particular for production of TNFα. A variety of pathophysiologic processes that directly or indirectly could contribute to deterioration of heart failure are influenced by immune activation, and specifically by TNFα:

a) TNF is detrimental for endothelial function and peripheral blood flow. In the short term TNF can up-regulate iNOS (as is seen in sepsis) and thereby contribute to vasodilation, but chronically TNF may in particular down-regulate cNOS. We have found a strong inverse correlation between the levels of TNF and the peak leg blood flow response to ischaemia ($r=-0.7$, $p<0.0001$). Impaired peripheral blood flow is closely linked to exercise capacity in CHF patients - particularly in cachectic patients.

b) Impaired peripheral blood flow is also an important component of the insulin resistance syndrome that we have shown to be present in CHF - insulin resistance appears to be a cause of energy depletion in the peripheral musculature.

c) TNF has negative inotropic effects on the heart (Starr *et al* (1995)

Shock 3(5), 380-384.

d) The immune activation status in CHF is closely linked to the hormonal catabolic/anabolic balance in CHF patients (Anker *et al* (1997) *J Am Coll Cardiol* **30**, 997-1001).

e) TNF is the strongest correlate of the degree of weight loss in cachectic CHF patients.

f) TNF could trigger cell apoptosis - not only in the heart, but particularly also in the periphery. This could lead to tissue dysfunction, and finally to specific and/or general tissue wasting. General wasting is then closely related to impaired prognosis in CHF.

Endotoxin (lipopolysaccharide; LPS) signalling may be mediated through the CD14 molecule, as discussed, for example, in Anker *et al* (1997) *Am J Cardiol* **79**, 1426-1430, Wright (1991) Multiple receptors for endotoxin *Curr Opin Immunol* **3**, 83-90 and Ulevitch & Tobias (1995) Receptor-dependent mechanisms of cell stimulation by bacterial endotoxin *Ann Rev Immunol* **13**, 437-457.

No one has previously proposed that an agent that is able to inhibit the response by a cell to endotoxin (lipopolysaccharide; LPS) be useful in the management of patients with either acute or chronic heart failure.

Through multiple pathways immune activation is detrimental for heart failure. We show here that endotoxin is raised in oedematous compared to non-oedematous heart failure, and propose that inhibiting the response by cells to endotoxin (lipopolysaccharide; LPS) may lead to improved immune status, which could through multiple mechanisms improve the

prognosis and clinical status of patients in the short and long term.

A first aspect of the invention provides a method of treating, preventing or ameliorating chronic heart failure or acute heart failure in a patient the method comprising administering to the patient an effective amount of a compound that is able to inhibit the response by a cell to endotoxin (LPS).

A second aspect of the invention provides a method of treating, preventing or ameliorating endotoxin-mediated immune activation in acute or chronic heart failure in a patient the method comprising administering to the patient an effective amount of a compound that is able to inhibit the response by a cell to endotoxin (LPS).

The following classes of patients in particular may benefit from treatment

1. Patients with acute heart failure (decompensated chronic heart failure, myocardial infarction).
2. Any decompensated heart failure patients with evidence of peripheral oedema.
3. Patients with severe heart failure (NYHA class III or IV) or with cardiac cachexia.
4. Stable CHF patients if any deterioration occurs, for example patients with a history of decompensation phases.

It is preferred that the patient has peripheral and/or bowel oedema.

Typically, in relation to the treatment of acute heart failure, the compound may be administered following myocardial infarction.

Acute heart failure is most frequently characterised by the presence of shortness of breath and oedema. It is most frequently treated by adjusting diuretics. It will be appreciated that the methods of the invention may be used in conjunction with other treatments for acute or chronic heart failure, for example treatment with diuretics. Thus, a further aspect of the invention is a method or use of the invention (as described below) wherein a diuretic is administered to the patient. The diuretic may be administered to the patient before, after or concurrently with the compound of the method or use of the invention.

The compound may decrease the endotoxin (LPS) sensitivity of, for example, immune system cells and thereby decrease the cytokine production by these cells, for example it may decrease the production of TNFα. Thus the compound may be a compound known to act as an immune regulatory drug, for example a corticosteroid (steroidal anti-inflammatory) or a leukotriene inhibitor. It is preferred that the compound acts directly on a cell that is stimulated directly by endotoxin. It is further preferred that the compound acts to modulate signalling within a cell caused by endotoxin binding to or otherwise interacting with that cell.

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A further aspect of the invention provides a method of treating, preventing or ameliorating chronic heart failure or acute heart failure in a patient the method comprising administering to the patient an effective amount of a corticosteriod or an antibody able to bind the CD14 receptor.

A further aspect of the invention provides a method of treating, preventing or ameliorating endotoxin-mediated immune activation in acute or chronic heart failure in a patient the method comprising administering to the patient an effective amount of a corticosteriod or an antibody able to bind the CD14 receptor.

The compound may act to reduce the level of receptors through which endotoxin (LPS) acts, for example CD14 receptors, for example by reducing the formation of receptors, for example CD14 receptors. Thus, the compound may interfere with the transcription or translation of the gene encoding the CD14 receptor. It may be an antisense compound, for example directed against the mRNA encoding the CD14 receptor. The CD14 receptor sequence is reported in, for example, Ferrero E & Goyert SM (1988) Nucleotide sequence of the gene encoding the monocyte differentiation antigen, CD14. *Nucleic Acids Res* **16(9)**, 4173. Thus, the compound may inhibit signalling *via* the CD14 receptor.

Antisense oligonucleotides are single-stranded nucleic acid, which can specifically bind to a complementary nucleic acid sequence. By binding to

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the appropriate target sequence, an RNA-RNA, a DNA-DNA, or RNA-DNA duplex is formed. These nucleic acids are often termed "antisense" because they are complementary to the sense or coding strand of the gene. Recently, formation of a triple helix has proven possible where the oligonucleotide is bound to a DNA duplex. It was found that oligonucleotides could recognise sequences in the major groove of the DNA double helix. A triple helix was formed thereby. This suggests that it is possible to synthesise a sequence-specific molecules which specifically bind double-stranded DNA *via* recognition of major groove hydrogen binding sites.

By binding to the target nucleic acid, the above oligonucleotides can inhibit the function of the target nucleic acid. This could, for example, be a result of blocking the transcription, processing, poly(A)addition, replication, translation, or promoting inhibitory mechanisms of the cells, such as promoting RNA degradations.

Antisense oligonucleotides are prepared in the laboratory and then introduced into cells, for example by microinjection or uptake from the cell culture medium into the cells, or they are expressed in cells after transfection with plasmids or retroviruses or other vectors carrying an antisense gene. Antisense oligonucleotides were first discovered to inhibit viral replication or expression in cell culture for Rous sarcoma virus, vesicular stomatitis virus, herpes simplex virus type 1, simian virus and influenza virus. Since then, inhibition of mRNA translation by antisense oligonucleotides has been studied extensively in cell-free systems including rabbit reticulocyte lysates and wheat germ extracts. Inhibition of viral function by antisense oligonucleotides has been demonstrated *in*

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vitro using oligonucleotides which were complementary to the AIDS HIV retrovirus RNA (Goodchild, J. 1988 "Inhibition of Human Immunodeficiency Virus Replication by Antisense Oligodeoxynucleotides", *Proc. Natl. Acad. Sci. (USA)* **85**(15), 5507-11). The Goodchild study showed that oligonucleotides that were most effective were complementary to the poly(A) signal; also effective were those targeted at the 5′ end of the RNA, particularly the cap and 5′ untranslated region, next to the primer binding site and at the primer binding site. The cap, 5′ untranslated region, and poly(A) signal lie within the sequence repeated at the ends of retrovirus RNA (R region) and the oligonucleotides complementary to these may bind twice to the RNA.

Oligonucleotides are subject to being degraded or inactivated by cellular endogenous nucleases. To counter this problem, it is possible to use modified oligonucleotides, eg having altered internucleotide linkages, in which the naturally occurring phosphodiester linkages have been replaced with another linkage. For example, Agrawal *et al* (1988) *Proc. Natl. Acad. Sci. USA* **85**, 7079-7083 showed increased inhibition in tissue culture of HIV-1 using oligonucleotide phosphoramidates and phosphorothioates. Sarin *et al* (1988) *Proc. Natl. Acad. Sci. USA* **85**, 7448-7451 demonstrated increased inhibition of HIV-1 using oligonucleotide methylphosphonates. Agrawal *et al* (1989) *Proc. Natl. Acad. Sci. USA* **86**, 7790-7794 showed inhibition of HIV-1 replication in both early-infected and chronically infected cell cultures, using nucleotide sequence-specific oligonucleotide phosphorothioates. Leither *et al* (1990) *Proc. Natl. Acad. Sci. USA* **87**, 3430-3434 report inhibition in tissue culture of influenza virus replication by oligonucleotide phosphorothioates.

Oligonucleotides having artificial linkages have been shown to be resistant to degradation *in vivo*. For example, Shaw *et al* (1991) in *Nucleic Acids Res.* **19**, 747-750, report that otherwise unmodified oligonucleotides become more resistant to nucleases *in vivo* when they are blocked at the 3′ end by certain capping structures and that uncapped oligonucleotide phosphorothioates are not degraded *in vivo*.

A detailed description of the H-phosphonate approach to synthesising oligonucleoside phosphorothioates is provided in Agrawal and Tang (1990) *Tetrahedron Letters* **31**, 7541-7544, the teachings of which are hereby incorporated herein by reference. Syntheses of oligonucleoside methylphosphonates, phosphorodithioates, phosphoramidates, phosphate esters, bridged phosphoramidates and bridge phosphorothioates are known in the art. See, for example, Agrawal and Goodchild (1987) *Tetrahedron Letters* **28**, 3539; Nielsen *et al* (1988) *Tetrahedron Letters* **29**, 2911; Jager *et al* (1988) *Biochemistry* **27**, 7237; Uznanski *et al* (1987) *Tetrahedron Letters* **28**, 3401; Bannwarth (1988) *Helv. Chim. Acta.* **71**, 1517; Crosstick and Vyle (1989) *Tetrahedron Letters* **30**, 4693; Agrawal *et al* (1990) *Proc. Natl. Acad. Sci. USA* **87**, 1401-1405, the teachings of which are incorporated herein by reference. Other methods for synthesis or production also are possible. In a preferred embodiment the oligonucleotide is a deoxyribonucleic acid (DNA), although ribonucleic acid (RNA) sequences may also be synthesised and applied.

The oligonucleotides useful in the invention preferably are designed to resist degradation by endogenous nucleolytic enzymes. *In vivo* degradation of oligonucleotides produces oligonucleotide breakdown products of reduced

length. Such breakdown products are more likely to engage in non-specific hybridization and are less likely to be effective, relative to their full-length counterparts. Thus, it is desirable to use oligonucleotides that are resistant to degradation in the body and which are able to reach the targeted cells. The present oligonucleotides can be rendered more resistant to degradation *in vivo* by substituting one or more internal artificial internucleotide linkages for the native phosphodiester linkages, for example, by replacing phosphate with sulphur in the linkage. Examples of linkages that may be used include phosphorothioates, methylphosphonates, sulphone, sulphate, ketyl, phosphorodithioates, various phosphoramidates, phosphate esters, bridged phosphorothioates and bridged phosphoramidates. Such examples are illustrative, rather than limiting, since other internucleotide linkages are known in the art. See, for example, Cohen, (1990) *Trends in Biotechnology*. The synthesis of oligonucleotides having one or more of these linkages substituted for the phosphodiester internucleotide linkages is well known in the art, including synthetic pathways for producing oligonucleotides having mixed internucleotide linkages.

Oligonucleotides can be made resistant to extension by endogenous enzymes by "capping" or incorporating similar groups on the 5′ or 3′ terminal nucleotides. A reagent for capping is commercially available as Amino-Link II™ from Applied BioSystems Inc, Foster City, CA. Methods for capping are described, for example, by Shaw *et al* (1991) *Nucleic Acids Res.* **19**, 747-750 and Agrawal *et al* (1991) *Proc. Natl. Acad. Sci. USA* **88(17)**, 7595-7599, the teachings of which are hereby incorporated herein by reference.

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A further method of making oligonucleotides resistant to nuclease attack is for them to be "self-stabilised" as described by Tang *et al* (1993) *Nucl. Acids Res.* 21, 2729-2735 incorporated herein by reference. Self-stabilised oligonucleotides have hairpin loop structures at their 3' ends, and show increased resistance to degradation by snake venom phosphodiesterase, DNA polymerase I and fetal bovine serum. The self-stabilised region of the oligonucleotide does not interfere in hybridization with complementary nucleic acids, and pharmacokinetic and stability studies in mice have shown increased *in vivo* persistence of self-stabilised oligonucleotides with respect to their linear counterparts.

It is preferred that the antisense reagent is able to bind to nucleic acid encoding a receptor that mediates endotoxin (LPS) signalling, for example CD14.

The antisense compound may be administered systemically. The oligonucleotides also can be incorporated into an implantable device which when placed at the desired site, permits the oligonucleotides to be released into the surrounding locus. For example, implants made of biodegradable materials such as polyanhydrides, polyorthoesters, polylactic acid and polyglycolic acid and copolymers thereof, collagen, and protein polymers, or non-biodegradable materials such as ethylenevinyl acetate (EVAc), polyvinyl acetate, ethylene vinyl alcohol, and derivatives thereof can be used to locally deliver the oligonucleotides. The oligonucleotides can be incorporated into the material as it is polymerised or solidified, using melt or solvent evaporation techniques, or mechanically mixed with the material. In one embodiment, the oligonucleotides are mixed into or applied onto

coatings for implantable devices such as dextran coated silica beads, stents, or catheters.

The dose of oligonucleotides is dependent on the size of the oligonucleotides and the purpose for which is it administered. In general, the range is calculated based on the surface area of tissue to be treated. The effective dose of oligonucleotide is somewhat dependent on the length and chemical composition of the oligonucleotide but is generally in the range of about 30 to 3000 μg per square centimetre of tissue surface area.

The oligonucleotides may be administered to the patient systemically for both therapeutic and prophylactic purposes. The oligonucleotides may be administered by any effective method, for example, parenterally (eg intravenously, subcutaneously, intramuscularly) or by oral, nasal or other means which permit the oligonucleotides to access and circulate in the patient's bloodstream. Oligonucleotides administered systemically preferably are given in addition to locally administered oligonucleotides, but also have utility in the absence of local administration. A dosage in the range of from about 0.1 to about 10 grams per administration to an adult human generally will be effective for this purpose.

It will be appreciated that it may be desirable to target the antisense oligonucleotides to immune system cells, for example mononuclear phagocytes. This may be achieved by using antisense oligonucleotides which are in association with a molecule which selectively directs the antisense oligonucleotide to the immune system cells, for example mononuclear phagocytes. For example, the antisense oligonucleotide may be associated with an antibody or antibody like molecule which selectively

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binds an antigen present on appropriate immune system cells. Such antigens are well known to those skilled in the art. By "associated with" we mean that the antisense oligonucleotide and the immune cell-directing entity are so associated that the immune cell-directing entity is able to direct the antisense oligonucleotide to the immune system cells, for mononuclear phagocytes.

It will be appreciated that antisense agents also include larger molecules which bind to the receptor, for example CD14 mRNA or genes and substantially prevent expression of the receptor, for example CD14 mRNA or genes and substantially prevent expression of said receptor, for example CD14 protein. Thus, expression of an antisense molecule which is substantially complementary to the receptor, for example CD14 mRNA is envisaged as part of the invention.

The said larger molecules may be expressed from any suitable genetic construct as is described below and delivered to the patient. Typically, the genetic construct which expresses the antisense molecule comprises at least a portion of the said receptor, for example CD14, mRNA or gene operatively linked to a promoter which can express the antisense molecule in the immune system cell. Promoters that may be active in immune system cells, for example mononuclear phagocytic cells will be known to those skilled in the art, and may include promoters for ubiquitously expressed, for example housekeeping genes.

Although the genetic construct can be DNA or RNA it is preferred if it is DNA.

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Preferably, the genetic construct is adapted for delivery to a human cell.

Means and methods of introducing a genetic construct into a cell in an animal body are known in the art. For example, the constructs of the invention may be introduced into the tumour cells by any convenient method, for example methods involving retroviruses, so that the construct is inserted into the genome of the tumour cell. For example, in Kuriyama *et al* (1991) *Cell Struc. and Func.* **16**, 503-510 purified retroviruses are administered. Retroviruses provide a potential means of selectively infecting cancer cells because they can only integrate into the genome of dividing cells; most normal cells surrounding cancers are in a quiescent, non-receptive stage of cell growth or, at least, are dividing much less rapidly than the tumour cells. Retroviral DNA constructs which encode said antisense agents may be made using methods well known in the art. To produce active retrovirus from such a construct it is usual to use an ecotropic psi2 packaging cell line grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal calf serum (FCS). Transfection of the cell line is conveniently by calcium phosphate co-precipitation, and stable transformants are selected by addition of G418 to a final concentration of 1 mg/ml (assuming the retroviral construct contains a *neo*^{R} gene). Independent colonies are isolated and expanded and the culture supernatant removed, filtered through a 0.45 μm pore-size filter and stored at -70°. For the introduction of the retrovirus into the tumour cells, it is convenient to inject directly retroviral supernatant to which 10 μg/ml Polybrene has been added. For tumours exceeding 10 mm in diameter it is appropriate to inject between 0.1 ml and 1 ml of retroviral supernatant; preferably 0.5 ml.

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Alternatively, as described in Culver *et al* (1992) *Science* 256, 1550-1552, cells which produce retroviruses are injected into the tumour. The retrovirus-producing cells so introduced are engineered to actively produce retroviral vector particles so that continuous productions of the vector occurred within the tumour mass *in situ*. Thus, proliferating tumour cells can be successfully transduced *in vivo* if mixed with retroviral vector-producing cells.

Targeted retroviruses are also available for use in the invention; for example, sequences conferring specific binding affinities may be engineered into preexisting viral *env* genes (see Miller & Vile (1995) *Faseb J.* 9, 190-199 for a review of this and other targeted vectors for gene therapy).

Other methods involve simple delivery of the construct into the cell for expression therein either for a limited time or, following integration into the genome, for a longer time. An example of the latter approach includes (preferably tumour-cell-targeted) liposomes (Nässander *et al* (1992) *Cancer Res.* 52, 646-653).

Immunoliposomes (antibody-directed liposomes) are especially useful in targeting to cancer cell types which over-express a cell surface protein for which antibodies are available. For the preparation of immuno-liposomes MPB-PE (N-[4-(p-maleimidophenyl)butyryl]-phosphatidylethanolamine) is synthesised according to the method of Martin & Papahadjopoulos (1982) *J. Biol. Chem.* 257, 286-288. MPB-PE is incorporated into the liposomal bilayers to allow a covalent coupling of the antibody, or fragment thereof,

to the liposomal surface. The liposome is conveniently loaded with the DNA or other genetic construct of the invention for delivery to the target cells, for example, by forming the said liposomes in a solution of the DNA or other genetic construct, followed by sequential extrusion through polycarbonate membrane filters with 0.6 μm and 0.2 μm pore size under nitrogen pressures up to 0.8 MPa. After extrusion, entrapped DNA construct is separated from free DNA construct by ultracentrifugation at 80 000 x g for 45 min. Freshly prepared MPB-PE-liposomes in deoxygenated buffer are mixed with freshly prepared antibody (or fragment thereof) and the coupling reactions are carried out in a nitrogen atmosphere at 4°C under constant end over end rotation overnight. The immunoliposomes are separated from unconjugated antibodies by ultracentrifugation at 80 000 x g for 45 min. Immunoliposomes may be injected intraperitoneally or directly into the tumour.

Other methods of delivery include adenoviruses carrying external DNA via an antibody-polylysine bridge (see Curiel *Prog. Med. Virol.* **40**, 1-18) and transferrin-polycation conjugates as carriers (Wagner *et al* (1990) *Proc. Natl. Acad. Sci. USA* **87**, 3410-3414). In the first of these methods a polycation-antibody complex is formed with the DNA construct or other genetic construct of the invention, wherein the antibody is specific for either wild-type adenovirus or a variant adenovirus in which a new epitope has been introduced which binds the antibody. The polycation moiety binds the DNA *via* electrostatic interactions with the phosphate backbone. The adenovirus, because it contains unaltered fibre and penton proteins, is internalised into the cell and carries into the cell with it the DNA construct of the invention. It is preferred if the polycation is polylysine.

The DNA may also be delivered by adenovirus wherein it is present within the adenovirus particle, for example, as described below.

In the second of these methods, a high-efficiency nucleic acid delivery system that uses receptor-mediated endocytosis to carry DNA macromolecules into cells is employed. This is accomplished by conjugating the iron-transport protein transferrin to polycations that bind nucleic acids. Human transferrin, or the chicken homologue conalbumin, or combinations thereof is covalently linked to the small DNA-binding protein protamine or to polylysines of various sizes through a disulfide linkage. These modified transferrin molecules maintain their ability to bind their cognate receptor and to mediate efficient iron transport into the cell. The transferrin-polycation molecules form electrophoretically stable complexes with DNA constructs or other genetic constructs of the invention independent of nucleic acid size (from short oligonucleotides to DNA of 21 kilobase pairs). When complexes of transferrin-polycation and the DNA constructs or other genetic constructs of the invention are supplied to the tumour cells, a high level of expression from the construct in the cells is expected.

High-efficiency receptor-mediated delivery of the DNA constructs or other genetic constructs of the invention using the endosome-disruption activity of defective or chemically inactivated adenovirus particles produced by the methods of Cotten *et al* (1992) *Proc. Natl. Acad. Sci. USA* **89**, 6094-6098 may also be used. This approach appears to rely on the fact that adenoviruses are adapted to allow release of their DNA from an endosome without passage through the lysosome, and in the presence of, for example

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transferrin linked to the DNA construct or other genetic construct of the invention, the construct is taken up by the cell by the same route as the adenovirus particle.

This approach has the advantages that there is no need to use complex retroviral constructs; there is no permanent modification of the genome as occurs with retroviral infection; and the targeted expression system is coupled with a targeted delivery system, thus reducing toxicity to other cell types.

It will be appreciated that "naked DNA" and DNA complexed with cationic and neutral lipids may also be useful in introducing the DNA into cells of the patient to be treated. Non-viral approaches to gene therapy are described in Ledley (1995) *Human Gene Therapy* **6**, 1129-1144.

Alternative targeted delivery systems are also known such as the modified adenovirus system described in WO 94/10323 wherein, typically, the DNA is carried within the adenovirus, or adenovirus-like, particle. Michael *et al* (1995) *Gene Therapy* **2**, 660-668 describes modification of adenovirus to add a cell-selective moiety into a fibre protein. Mutant adenoviruses which replicate selectively in p53-deficient human tumour cells, such as those described in Bischoff *et al* (1996) *Science* **274**, 373-376 are also useful for delivering the genetic construct of the invention to a cell. Thus, it will be appreciated that a further aspect of the invention provides a virus or virus-like particle comprising a genetic construct of the invention. Other suitable viruses or virus-like particles include HSV, AAV, vaccinia and parvovirus.

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In a further embodiment the agent which is able to inhibit the response by a cell to endotoxin (LPS) is a ribozyme capable of cleaving targeted receptor, for example CD14, RNA or DNA. A gene expressing said ribozyme may be administered in substantially the same and using substantially the same vehicles as for the antisense molecules.

Ribozymes which may be encoded in the genomes of the viruses or virus-like particles herein disclosed are described in Cech and Herschlag "Site-specific cleavage of single stranded DNA" US 5,180,818; Altman *et al* "Cleavage of targeted RNA by RNAse P" US 5,168,053, Cantin *et al* "Ribozyme cleavage of HIV-1 RNA" US 5,149,796; Cech *et al* "RNA ribozyme restriction endoribonucleases and methods", US 5,116,742; Been *et al* "RNA ribozyme polymerases, dephosphorylases, restriction endonucleases and methods", US 5,093,246; and Been *et al* "RNA ribozyme polymerases, dephosphorylases, restriction endoribonucleases and methods; cleaves single-stranded RNA at specific site by transesterification", US 4,987,071, all incorporated herein by reference.

It will be appreciated that it may be desirable that the antisense molecule or ribozyme is expressed from a immune system cell-specific promoter element.

The genetic constructs described above can be prepared using methods well known in the art.

The compound may inhibit signalling *via* the receptor, for example the CD14 receptor. The compound may be an antibody that binds to CD14

and reduces its signalling activity. A suitable antibody may be described in US 5,730,980.

It is preferred that the compound is able to substantially reduce the amount of immune mediators produced in response to the presence of endotoxin (LPS).

It will be appreciated that the compound administered to the patient may be a single chemical species, or it may be a mixture of two or more chemical species.

The compound may be administered to the patient in any suitable form or in any suitable way. The compound or a formulation thereof may be administered by any conventional method including oral and by injection (in particular, intravascular injection). The treatment may consist of a single dose or a plurality of doses over a period of time.

Chronic use is suggested in any patient who is at increased risk of myocardial infarction (i.e. any patient with coronary artery disease - all at risk for acute heart failure) or in any patient with chronic heart failure (at risk for decompensation and cachexia development).

While it is possible for the compound to be administered alone, it is preferable to present it as a pharmaceutical formulation, together with one or more acceptable carriers. The carrier(s) must be "acceptable" in the

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sense of being compatible with the compound and not deleterious to the recipients thereof.

The formulations may conveniently be presented in unit dosage form and may be prepared by any of the methods well known in the art of pharmacy. Such methods include the step of bringing into association the compound (active ingredient; for example a corticosteroid or an antibody capable of binding CD14) with the carrier which constitutes one or more accessory ingredients. In general the formulations are prepared by uniformly and intimately bringing into association the active ingredient with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product.

Formulations in accordance with the present invention suitable for oral administration may be presented as discrete units such as capsules, sachets or tablets, each containing a predetermined amount of the active ingredient; as a powder or granules; as a solution or a suspension in an aqueous liquid or a non-aqueous liquid; or as an oil-in-water liquid emulsion or a water-in-oil liquid emulsion. The active ingredient may also be present as a bolus electuary or paste.

A tablet may be made by compression or moulding, optionally with one or more accessory ingredients. Compressed tablets may be prepared by compressing in a suitable machine the active ingredient in a free-flowing form such as a powdered or granules, optionally mixed with a binder (eg

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povidone, gelatin, hydroxypropylmethyl cellulose), lubricant, inert diluent, preservative, disintegrant (eg sodium starch glycollate, cross-linked povidone, cross-linked sodium carboxymethyld cellulose), surface-active or dispersing agent. Moulded tablets may be made by moulding in a suitable machine a mixture of the powdered compound moistened with an inert liquid diluent. The tablets may optionally be coated or scored and may be formulated so as to provide slow or controlled release of the active ingredient therein using, for example, hydroxypropylmethylcellulose in varying proportions to provide desired release profile.

Formulations suitable for parenteral administration include aqueous and non-aqueous sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats and solutes which render the formation isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents. The formulations may be presented in unit-dose or multi-dose containers, for example sealed ampoules and vials, and may be stored in a freeze-dried (lyophilised) condition requiring only the addition of the sterile liquid carrier, for example water for injections, immediately prior to use.

Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets of the kind previously described.

Preferred unit dosage formulations are those containing a daily dose or unit, daily sub-dose or an appropriate fraction thereof, of an active ingredient.

It should be understood that in addition to the ingredients particularly mentioned above the formulations of this invention may include other agents conventional in the art having regard to the type of formulation in question, for example those suitable for oral administration may include flavouring agents.

It will be appreciated that intravascular administration may be particularly desirable in the treatment of acute heart failure, for example where there is a desire for a quicker onset of action.

A third aspect of the invention provides use of a compound that is able to inhibit the response by a cell to endotoxin (LPS) in the manufacture of a medicament for treating, preventing or ameliorating endotoxin-mediated immune activation in acute or chronic heart failure in a patient. Preferences for the said compound are as set out above.

A fourth aspect of the invention provides a pharmaceutical formulation comprising a compound as defined above and a diuretic. A further aspect of the invention provides a kit of parts useful in treating, preventing or

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ameliorating acute or chronic heart failure comprising a compound as defined above and a diuretic.

Suitable diuretics are known to those skilled in the art and are described, for example in Martindale The Extra Pharmacopoeia, 31st Edition.

A fifth aspect of the invention provides any novel method of treating, preventing or ameliorating acute or chronic heart failure as herein disclosed.

The invention will now be described by reference to the following Examples and Figures:

Figure 1: Plasma levels of endotoxin, TNFα and soluble CD14 in patients with chronic heart failure (CHF) with and without peripheral edema compared to healthy volunteers (mean $\pm$standard error of the mean).

Figure 2: Effect of intensified diuretic treatment on plasma endotoxin levels in 10 CHF patients with peripheral edema (box plot displaying the 10th, 25th, 50th and 90th percentiles).

Example 1: Endotoxin and Immune Activation in Chronic Heart Failure.

Summary

Background: This study was designed to test the hypothesis that endotoxemia occurs during the congestive phase of CHF. Immune activation in chronic heart failure (CHF) patients may be secondary to endotoxin action.

Methods: We studied 20 CHF patients with recent onset of moderate to severe peripheral oedema secondary to cardiac congestion (age 64±2 y, NYHA class 3.3±0.1, mean±SEM) and compared them to 20 stable CHF patients (63±4 y, NYHA 2.6±0.2), and 14 healthy control subjects (55±4 y, ANOVA p=0.28). Blood samples for endotoxin measurements (LAL test, normal level <0.50 IU/mL) were collected in endotoxin free tubes. Biochemical markers of endotoxemia and inflammation, several cytokines and cell membrane proteins associated with immune activation were also measured. Ten patients were restudied within 1 week of complete resolution of oedema (5 patients survived >6 months and were restudied again).

Findings: Endotoxin levels were increased in oedematous CHF patients (0.74±0.10 IU/mL) as compared to stable CHF (0.37±0.05 IU/mL, p=0.0009) and controls (0.46±0.05 IU/mL, p=0.02); LPS binding protein (LBP) did not differ between groups. Compared to controls and stable CHF, oedematous CHF had highest levels of c-reactive protein (CRP, ANOVA p<0.003), tumor necrosis factor (TNF)-α (p<0.001), soluble (s) TNF receptor (-R)1 (p<0.001), sTNF-R2 (p<0.01), interleukin-6 (p<0.003), and sCD14 (p<0.001). Endotoxin levels correlated with sCD14 (r=0.30, p<0.03). CRP levels correlated with

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procalcitonin (r=0.74, p<0.0001), TNF-α (r=0.50, p=0.001), TNF-R1 (r=0.67, p<0.0001), and TNF-R2 (r=0.61, p<0.0001). FACS analyses revealed similar CD4/8 ratios in all groups, despite significantly reduced CD4 (p<0.02) and elevated CD8/25 (p<0.05) in CHF-oedema. Diuretic treatment with resolution of oedema resulted in normalisation of endotoxin levels after 23±8 days (n=10; 0.84±0.16 to 0.45±0.07 IU/mL, p<0.05), but cytokines remained elevated and LBP unchanged. After freedom of oedema >3 months endotoxin levels remained stable and normal (p=0.45, n=5), and TNF-α had decreased (39.6±5.5 to 31.0±2.5 pg/mL, p=0.079).

Interpretation: Elevated levels of endotoxin and cytokines without a concomitant increase in LBP are found in CHF patients during an acute oedematous exacerbation. Elevated endotoxin levels are normalised by intensified diuretic treatment, whereas normalisation of TNF-α levels is delayed. These data provide evidence for a role of endotoxin as a potential cause of immune activation in patients with congestive heart failure.

The results show that LPS is raised in oedematous CHF, but normal in non-oedematous heart failure patients. The increased LPS levels are linked to raised cytokine levels. Diuretic treatment reduces LPS levels. This suggests that oedema may causally be linked to elevated LPS levels. After treating the oedema, cytokine levels (TNF etc.) but also levels of soluble CD14 (a marker of cell - LPS interaction) do not fall immediately. The cytokine levels fall only after a longer period of clinical stability. This suggests that LPS sensitivity may be abnormal in subjects after a

phase of clinical instability, i.e. despite a "normal" level of LPS the interaction with immunological cells is still intensive (sCD14 is high) and cytokine production is still increased. LPS binding protein was not increased in any patient group.

Patients with chronic heart failure (CHF) exhibit immune activation which may be related to generalised body wasting (ie cardiac cachexia) [1,2]. Based on the finding of increased expression of tumor necrosis factor-α (TNF-α) in cardiac tissue of CHF patients undergoing heart transplantation the failing heart itself has been suggested as the cause of immune activation [3]. To date no link between a pathogenic process and cytokine activation in heart failure has been documented, either in patients with heart failure or animal models. The precise stimulus for the increased cytokine production seen in CHF patients remains unknown.

We have previously suggested that bacterial endotoxin, lipopolysaccharide (LPS), contributes to immune activation in CHF [4]. Acute venous congestion could cause immune activation *via* several mechanisms. Regional hypoxia could facilitate the generation of oxygen free radicals and altered gut permeability may lead to bacterial or LPS translocation. Alternatively, lung infection may be present. These events may increase LPS plasma levels and trigger increased cytokine production. LPS is bound by a serum protein termed LPS binding protein (LBP) [5], and it recently has been shown that the ratio of LPS to LBP is crucial for the immunostimulatory effects of LPS [6]. LBP levels *in vivo* can vary substantially due to transcriptional activation [7]. We have recently shown that high concentrations of LBP, as seen during the acute phase response,

can completely block LPS effects *in vitro* and in a murine sepsis model [8]. Furthermore, in our previous study [4] patients with high soluble (s) CD14 levels (indicative of endotoxin-cell interaction and shedding of CD14 from the cell membrane [9]) showed markedly increased levels of TNF-α, sTNF receptor (R)-1 and -2, and intercellular adhesion molecule-1 (ICAM-1). A recent report documented that sCD14 alone can stimulate immune cells to produce cytokines [10].

In the present study, we measured endotoxin, LBP and sCD14 and related levels to markers of cellular and humoral immune activation in CHF patients and healthy volunteers. Among CHF patients bowel wall oedema that could cause altered gut permeability and bacterial (ie endotoxin) translocation is most likely to occur in patients with moderate to severe peripheral oedema. Thus, we compared patients with recent onset oedematous decompensation to stable non-oedematous CHF patients. In a subgroup of oedematous patients we assessed the effect of diuretic therapy, anticipating that such treatment would lead to a reduction of endotoxin.

METHODS

Fourteen healthy volunteers (age: 55±4 y) and 40 CHF patients (age: 63±3 y, $p=0.30$) were studied prospectively. The aetiology of CHF was ischaemic in 27 patients and idiopathic dilated cardiomyopathy in 13 patients. The diagnosis of CHF was based on symptomatic exercise intolerance, cardiomegaly, and documented left ventricular dysfunction (all patients had a left ventricular ejection fraction of less than 40%). No subject had clinical signs of infection, rheumatoid arthritis, or cancer.

Cardiac decompensation has been associated with the presence of bowel wall oedema secondary to venous congestion. We were not able to measure directly the degree of bowel wall oedema. The relationship between central haemodynamics and the pathophysiological alterations in CHF is weak [11,12]. In animal models there is a poor relationship between intracardiac pressures and intestinal perfusion [13]. Thus, we divided patients according to the presence or absence of a reliable marker of acute venous congestion due to cardiac failure, namely peripheral oedema.

Twenty CHF patients were clinically stable without evidence of peripheral oedema, and 20 patients presented with moderate to severe oedema to the outpatient clinic of the Royal Brompton Hospital in London, UK. The CHF patients were treated with diuretics (n=38), an angiotensin converting enzyme inhibitor (n=36), digoxin (n=14), aspirin (n=17), amiodarone (n=16) and nitrates (n=15) in varying combination. The clinical details of patients and controls are given in Table 1. Ten oedematous patients who lived close to our hospital (NYHA class IV: 5, class III: 5) were followed-up after treatment with increased doses of diuretics (increase of frusemide up to 120 mg/day, addition of bendrofluazide (2.5 or 5 mg od), and/or metolazone (5 or 10 mg od)). Of these patients three had to be admitted for 3 to 8 days for intravenous diuretic treatment. After 23±8 days these patients were restudied within 1 week after complete resolution of oedema (NYHA class after treatment: III - 6, II - 4; weight loss: 3.6±0.3 kg [range 2.5 to 5.0 kg]). Five patients regained clinical stability (NYHA class: III - 1, II - 4) and were restudied again 14 to 32 weeks (mean 21±3 weeks) after the initial

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investigation when they had been free of peripheral oedema for more then 3 months. The remaining 5 patients did not reach a longer-term stable clinical state again and died 2 to 8 months after the initial investigation without having been restudied. The research protocol was approved by the ethics committee of the Royal Brompton Hospital, and all patients and controls gave written informed consent.

Blood samples. Blood samples were collected on presentation in the outpatient clinic after supine rest for at least 15 min. An antecubital polyethylene catheter was inserted and 8 mL of venous blood were drawn into endotoxin free tubes (Endo Tube ET ®, Chromogenix AB, Sweden), and 30 mL of standard venous samples were taken for biochemical and cytokine measurements. After immediate centrifugation endotubes and plasma aliquots were stored at -80°C until analysis. In addition, 5 mL EDTA blood was taken to perform fluorescence activated cell sorting (FACS) analysis.

Assessment of endotoxin. Levels of endotoxin were measured by using a commercially available kit (Limulus Amebocyte Lysate QCL-1000 test kit, BioWhittaker Inc., Walkersville, USA). The normal level of endotoxin in this assay in healthy subjects is < 0.50 IU/mL. Endotoxin in the patient sample activates a protein in the Limulus amebocyte lysate, so that it possesses enzymatic activity. The activated enzyme catalyses the release of p-nitroaniline from a short synthetic peptide; p-nitroaniline can be detected by acidification with acetic acid, and measuring absorbance at 410 nm (sensitivity 0.03 IU/mL). The coefficient of variance for the LPS reproducibility with the LAL test kit is $<10\%$.

Cytokine and other analyses. LBP-levels were determined by an ELISA assay as described previously [14]. Total tumor necrosis factor (TNF)-α was measured with an ELISA test kit from Medgenix (Fleurus, Belgium; sensitivity 3.0 pg/mL; test not influenced by soluble TNF receptors). Soluble TNF receptors 1 (sTNF-R1; sensitivity 25 pg/mL), sTNF-R2 (sensitivity 2 pg/mL), and interleukin-6 (IL-6; sensitivity 0.0094 pg/mL, all kits: R&D Systems, Minneapolis, MN, USA), and sCD14 (IBL, Hamburg, Germany) were assessed by ELISA. Plasma procalcitonin (PCT) levels were measured by an immunoluminometric assay using two monoclonal antibodies (BRAHMS, Berlin, Germany) [15,16]. The normal level of PCT in this assay in healthy subjects is < 0.6 ng/ml.

FACS analysis. Whole blood samples were supplied for analysis in K-EDTA tubes (Vacutaner Systems, Falcon BD Oxford UK) and stained with fluorescently labeled monoclonal antibodies (Coulter Electronics, Luton UK) to determine peripheral lymphocyte phenotype and the proportion of CD25 receptor (CD25R) positive T cells. Briefly, a staining excess of antibody, determined by titration (data not shown), was aliquoted into 12 x 75 mm polypropylene tubes (Elkay, Hampshire UK). Two tubes were analysed for each patient sample point. The first contained control monoclonal mouse anti-human antibodies isotipically matched to the test antibodies in the second tube. The antibody-flurochrome conjugates used were CD3-PC5, CD4-FITC, CD8-ECD, CD25R-RD1. The Immunoprep formic acid lysed whole blood protocol was used in the multi-Q-prep (Coulter Electronics, Luton, UK).

Lymphocyte gating was set on forward versus side scatter dot plot and compensation established by combining single colour stained leukocyte populations. Four colour flow cytometric analysis was performed on the Coulter XL-MCL employing System II software.

Statistical analyses. Normality of distribution was assessed using the Kolmogorow Smirnov test. Unpaired Student's t-test, paired t-test, ANOVA with Fisher's post hoc test, and Mann-Whitney U test were used where appropriate. Data are presented as mean $\pm$ standard error of the mean. We also performed univariate correlation analyses to establish the relationship between variables. A probability value of $p<0.05$ was considered significant.

RESULTS

Baseline analyses. In Table 1 and 2 baseline clinical characteristic and humoral measurements are detailed. Between controls and stable-CHF patients only uric acid and aspartate aminotransferase levels were significantly different. Oedematous CHF patients had more severe disease and showed a variety of biochemical abnormalities.

Endotoxin levels were highest in CHF patients with peripheral oedema (0.74 ± 0.10 IU/mL) compared to CHF patients without oedema (0.37 ± 0.05 IU/mL, $p=0.0009$), and controls (0.46 ± 0.05 IU/mL, $p=0.02$) (Figure 1). Plasma levels of LBP were not statistically different between groups (stable CHF: 10.4 ± 1.2 μg/mL, oedematous CHF: 12.1 ± 1.3 μg/mL, controls: 9.6 ± 1.3 μg/mL), but there was an elevated LPS / log LBP ratio in the CHF patients with oedema (oedematous CHF:

0.75±0.11, stable CHF: 0.44±0.07, controls: 0.54±0.05, ANOVA $p=0.03$, oedematous CHF vs stable CHF: $p<0.01$). In oedematous CHF patients levels were highest for CRP (+107% vs stable CHF, $p<0.03$; +252% vs controls, $p<0.001$), TNF-α (+42% vs stable CHF, $p<0.001$; +49% vs controls, $p<0.001$, Figure 1), sTNF-R1 (+78% vs stable CHF, $p<0.006$; +171% vs controls, $p<0.0005$), sTNFR-R2 (+50% vs stable CHF, $p<0.03$; +115% vs controls, $p<0.001$), IL-6 (+241% vs stable CHF, $p<0.005$; +635% vs controls, $p<0.002$) and sCD14 (+16% vs stable CHF, $p<0.003$; +23% vs controls, $p<0.0003$, Figure 1). A trend toward increased PCT levels in oedematous CHF patients was noted (ANOVA: $p=0.073$).

Analysing the data of all subjects, there were significant correlations of sCD14 with endotoxin ($r=0.30$, $p=0.028$), as well as with TNF-α ($r=0.36$, $p=0.008$), sTNF-R1 ($r=0.46$, $p=0.0005$), and sTNF-R2 ($r=0.38$, $p<0.009$). CRP correlated with PCT ($r=0.74$, $p<0.0001$), TNF-α ($r=0.49$, $p=0.001$), sTNF-R1 ($r=0.67$, $p<0.0001$), and sTNF-R2 ($r=0.61$, $p<0.0001$), but not with endotoxin ($r=0.09$, $p=0.57$). Furthermore, PCT correlated with sTNF-R1 ($r=0.50$, $p=0.0001$) and sTNF-R2 ($r=0.53$, $p<0.0001$), but not with TNF-α ($r=0.25$, $p=0.07$) and endotoxin ($r=0.03$, $p=0.83$). There were neither simple correlations of creatinine or urea plasma levels and LPS at baseline, nor of changes of markers of kidney function over time vs the changes of LPS or cytokine concentrations over time (data not shown). Thus a bias due to latent abnormalities of kidney function seen in some oedematous patients is unlikely.

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FACS analyses. There was significantly less CD4 in oedematous CHF patients (35±6%) as compared to stable-CHF (51±4%, $p<0.007$) and healthy volunteers (47±2%, $p<0.03$), whereas CD4/25 (CHF-oedema 10.6±3.3%, stable-CHF 5.5±0.7%, Con 6.7±1.1%, $p>0.2$), CD8 (CHF-oedema 28±8%, stable-CHF 23±5%, Con 22±2%, $p>0.2$), and the CD4/8 ratio (CHF-oedema 2.6±0.9%, stable-CHF 3.3±0.8%, Con 2.5±0.3%, $p>0.2$) were not different between groups. CD8/25 was significantly higher in patients with CHF-oedema (11.6±4.0%) than in healthy volunteers (4.7±0.6%, $p<0.02$), but not stable-CHF (8.7±1.6, $p>0.2$).

Influence of diuretic treatment. Intensive diuretic treatment of CHF patients (n=10) resulted in weight reduction of 3.6±0.3 kg (range 2.5 to 5.0 kg), and improvement of the functional NYHA class of 9 of the 10 patients. In 8 of 10 patients a reduction of the endotoxin plasma concentration by 17 to 90% was observed (mean for all patients: -46%); the LPS levels fell from 0.84±0.16 to 0.45±0.07 IU/mL (n=10, $p<0.05$; Figure 2). In 2 patients with normal levels at baseline, endotoxin levels were found at the upper end of the normal range after diuretic treatment, i.e. below 0.50 IU/mL (+9% and +36% compared to baseline). Diuretic treatment did not affect plasma levels of TNF-α (baseline: 39.9±4.2 pg/mL, after: 40.2±4.1 pg/mL), sTNF-R1 (baseline: 2336±415 pg/mL, after: 2765±440 pg/mL), sTNF-R2 (baseline: 3751±378 pg/mL, after: 4029±437 pg/mL), IL-6 (baseline: 19.4±7.3 pg/mL, after: 18.3±7.6 pg/mL), sCD14 (baseline: 4474±70 ng/mL, after: 4430±241 ng/mL), or LBP (baseline: 10.3±1.2 μg/mL, after: 12.7±2.4 μg/mL) compared to baseline (n=10, all $p>0.20$). During further follow-up, 5

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patients could be restudied when they had been free of oedema >3 months. Endotoxin remained stable at visit 3 (after 21±3 weeks: 0.49±0.03 IU/mL) compared to the second visit of these 5 patients (after 19±7 days: 0.39±0.10 IU/mL, $p=0.45$), but TNF-α decreased (visit 2: 39.6±5.5 vs visit 3: 31.0±2.5 pg/mL, $p=0.079$).

We have shown that endotoxin levels as well as pro-inflammatory cytokines are elevated in patients with heart failure who have peripheral oedema. Elevated endotoxin levels were normalised by prolonged diuretic treatment. The endotoxemia in these patients was not associated with a strong acute phase response that would have induced an increased hepatic LBP synthesis and subsequent blocking of LPS-effects. These results support the suggestion that bacterial endotoxin may be an important stimulus of immune activation in patients with chronic heart failure.

The complex of endotoxin and endotoxin binding protein activates cells *via* the CD14 protein on the surface of mononuclear phagocytes stimulating the production of TNF-α and other cytokines [17,18]. Previous studies suggested that increased sCD14 levels might be related to endotoxemia [9], but this is the first study to document directly the significant relationship between endotoxin and sCD14. Shedded and therefore soluble CD14 receptors are thought to reflect the amount of endotoxin - cell interaction over prolonged time intervals. In contrast, endotoxin itself has a short plasma half-life time (in the range of 10 to 30 min). This may explain why sCD14 levels are more closely related to the cytokine levels than endotoxin levels, as shown here and previously [4]. PCT (procalcitonin) plasma levels have been suggested to be indicative of

systemic bacterial infections and are less prominent in endotoxemia [16], although the mechanisms are not clear. This study showed only a trend for raised PCT levels in oedematous CHF patients (ANOVA: $p<0.08$), and therefore only low grade bacteraemia, if at all, may be present. That conclusion is supported by results from FACS analysis, showing only ~~moderate changes in the pattern of cellular immune activation.~~ Additionally, the levels of endotoxin observed in this study were well below those otherwise seen in septic shock [19]. The CHF patients studied here had no sign of active infection, and the moderate increase of plasma endotoxin levels is in keeping with the hypothesis of a translocation process. Possibly, it is endotoxin itself rather than bacteria which translocates.

Although intensified diuretic therapy resulted in normalisation of endotoxin levels, treatment did not lead immediately to reduced cytokine plasma levels, which is in keeping with a previous study [20]. This may be due to a concentration effect due to the loss of up to 5 kg body water therefore concentrating plasma levels or due to prolonged activation of monocytes/macrophages following exposure to an endotoxin stimulus during a phase of clinical deterioration with increased venous congestion, ie "normalised" endotoxin levels may still cause increased cytokine production. Indeed, such an increased cellular LPS sensitivity has recently been documented for CHF patients with acute decompensation [21], and increased TNF-α releases at baseline and after endotoxin stimulation have recently been found in cardiomyocytes from cardiac transplantation recipients, particularly for those with heart failure of ischaemic aetiology [22]. Also the previously documented raised TNF-α

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levels in cardiac tissue of end-stage CHF patients [3] may be due to cardiomyocytes or tissue monocytes producing increased amounts of cytokines upon stimulation by LPS, either because these patients were decompensated or because the cardiomyocytes were hypersensitive. After a prolonged phase of clinical stability TNF-α plasma levels showed a strong trend to decrease back to normal, ie the normalisation of the relative cytokine secretion capacity may be a slow process.

Tolerance of monocytes/macrophages to endotoxin can be induced both *in vivo* and *in vitro* by endotoxin itself, and for instance it frequently occurs after severe injury [23]. One important mediator of LPS hyposensitivity is IL-10 [24]. Compared to controls, we previously found IL-10 to be lower in stable CHF patients [4]. Glucocorticoids are well known to be able to suppress LPS triggered immune activation [25], and for their general immuno suppressive effects they are considered standard in the treatment of transplant patients. Nevertheless, glucocorticoids are under certain circumstances also a prerequisite for an increased immune response [26]. In CHF patients we have recently shown that the cortisol/DHEA ratio is closely related to the degree of immune activation [27]. This marker of catabolic/anabolic balance is highest in cachectic CHF patients [2], who also demonstrate pronounced immune activation [1,2]. Increased cardiac wall stress and tissue hypoxia (both *via* local free radical generation and subsequent stimulation of the nuclear factor-kappaB pathway [28]) and hormonal catabolic/anabolic imbalance may cause immunological hypersensitivity, and endotoxin may thus be an important stimulus for cytokine production both in the heart and in the periphery. *In vitro* already low levels of LPS have detrimental effects on cardiomyocytes

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[29]. *In vivo* there may be a dynamic balance between heart function and immune activation in CHF patients [30]. Over time patients with frequent oedematous episodes may suffer most from the cardio-depressant [31,32] and metabolic [33,34] consequences of raised TNF-α levels, arguing for a tight control of the fluid balance of CHF patients.

In stable ambulatory patients Munger *et al* [35] have not been able to show a significant spill-over of cytokines from the heart, suggesting that cardiac production could not be the main source of the raised peripheral cytokine plasma levels. Supporting the importance of peripheral hypoxia, recently measures of increased oxidative stress have been found to correlate with sTNFR-1/2 levels [36]. We have shown that post-ischaemic peak leg blood flow in clinically stable CHF patients is inversely related to TNF-α plasma levels [37]. This may be due to a relationship between hypoxia and TNF-α production, or alternatively due to toxic effects of TNF-α on endothelial function [38]. Hypoxia *per se* may not be the most important cytokine trigger in CHF patients because of differences in the cytokine profile. Raised IL-6 plasma levels can be attributed to peripheral hypoxic conditions [39] that will certainly occur in CHF [40], but there is no report that hypoxia per se induces TNF-α, PCT, sTNF-R1 or sTNF-R2 [41]. Increased levels of soluble TNF-α receptors and particularly sCD14 are, in contrast, characteristic of endotoxin action, but not of hypoxic conditions [42].

CONCLUSION

This study demonstrates the presence of raised plasma endotoxin concentrations in patients with CHF and peripheral oedema. In the

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presence of unchanged levels of endotoxin binding protein this reflects a potentially pathogenic situation leading to cytokine induction. We show that normalisation of endotoxin levels can be achieved by intensified diuretic treatment. Bacterial endotoxin may be an important stimulus of immune activation in patients with chronic heart failure.

References:

1. Levine B, Kalman J, Mayer L, Fillit HM, Packer M. Elevated circulating levels of tumor necrosis factor in severe chronic heart failure. N Engl J Med 1990;323:236-241.

2. Anker SD, Swan JW, Chua TP, Ponikowski P, Harrington D, Kox WJ, Poole-Wilson PA, Coats AJS. Hormonal changes and catabolic/anabolic imbalance in chronic heart failure: The importance for cardiac cachexia. Circulation 1997;96:526-534.

3. Torre-Amione G, Kapadia S, Lee J, Durand J-B, Bies RD, Young JB, Mann DL. Tumor necrosis factor-α and tumor necrosis factor receptors in the failing human heart. Circulation 1996;93:704-711.

4. Anker SD, Egerer K, Volk H-D, Kox WJ, Poole-Wilson PA, Coats AJS. Elevated soluble CD14 receptors and and altert cytokines in chronic heart failure. Am J Cardiol 1997;79:1426-1430.

5. Schumann RR, Leong SR, Flaggs GW, Gray PW, Wright SD, Mathison JC, Tobias PS, Ulevitch RJ. Structure and function of lipopolysaccharide binding protein. Science 1990;249,1429-1431.

6. Tobias, PS, Soldau K, Iovine NM, Elsbach P, Weiss P. Lipopolysaccharide (LPS) binding proteins BPI and LBP form different types of complexes with LPS. J Biol Chem 1997;272: 18682-18685.

4

7. Schumann RR, Kirschning C, Unbehaun A, Aberle H, Knopf H-P, Ulevitch RJ, Herrmann, F. Lipopolysaccharide binding protein (LBP) is a secretory class 1 acute phase protein requiring binding of the transcription factor STAT-3, C/EBPβ, and AP-1. Mol Cell Biol 1996;16:3490-3503.

8. Lamping N, Dettmer R, Schröder NWJ, Pfeil D, Hallatschek W, Burger R, Schumann RR. LPS-binding protein protects mice from septic shock caused by LPS or gram-negative bacteria. J Clin Invest 1998;101:2065-2071.

9. Ziegler-Heitbrock, Ulevitch RJ. CD14: Cell surface receptor and differentiation marker. Immunology Today 1993;14:121-125.

10. Landmann R, Link S, Sausano S, Rajacic Z, Zimmerli W. Soluble CD14 activates monocytic cells ndependently of lipopolysaccharide. Infect Immun 1998;66:2264-2271.

11. Clark AL, Poole-Wilson PA, Coats AJ. Exercise limitation in chronic heart failure: central role of the periphery. J Am Coll Cardiol 1996;28:1092-1102.

12. Anker SD, Coats AJS. Metabolic, functional, and haemodynamic staging for CHF? Lancet 1996;348:1530-1531.

13. Sautner T, Wessely C, Riegler M, Sedivy R, Gotzinger P, Losert U, Roth E, Jakesz R, Fugger R. Early effects of catecholamine therapy on mucosal integrity, intestinal blood flow, and oxygen metabolism in porcine endotoxin shock. Ann Surg 1998;228:239-248.

14. Lamping N, Hoess A, Yu B, Park TC, Kirschning C, Pfeil D, Reuter D, Wright SD, Herrmann F, Schumann RR. Effect of site-directed mutagenisis of gasic residues (Arg 94, Lys 95, Lys 99) of lipopolysaccharide (LPS)-binding protein on binding and transfer of LPS and subsequent immune cell activation. J Immunol 1996;157:4648-4656.

4

15. Dandona P, Nix D, Wilson MF, Aljada A, Love J, Assicot M, Bohoun C. Procalcitonin increase after endotoxin injection in normal subjects. J Clin Endocrinol Metab 1994;79:1605-1608.

16. Assicot M, Gendrel D, Carsin H, Raymond J, Guilbaud J, Bohuon C. High serum procalcitonin concentrations in patients with sepsis and infection. Lancet 1993;341:515-518.

17. Wright SD. Multiple receptors for endotoxin. Curr Opin Immunol 1991;3:83-90.

18. Ulevitch RJ, Tobias PS. Receptor-dependent mechanisms of cell stimulation by bacterial endotoxin. Annu Rev Immunol 1995;13:437-457.

19. Gomez-Jimenez J, Salgado A, Mourelle M, Martin MC, Segura RM, Peracaula R, Moncada S. L-arginine: nitric oxide pathway in endotoxemia and human septic shock. Crit Care Med 1995;23:253-258.

20. Vanderheyden M, Kersschot E, Paulus WJ. Pro-inflammatory cytokines and endothelium-dependent vasodilation in the forearm. Serial assessment in patients with congestive heart failure. Eur Heart J 1998;19:747-752.

21. Vonhof S, Brost B, Stille-Siegener M, Grumbach IM, Kreuzer H, Figulla HR. Monocyte activation in congestive heart failure due to coronary artery disease and idiopathic dilated cardiomyopathy. Int J Cardiol 1998;63:237-244.

22. Wagner DR, McTiernan C, Sanders VJ, Feldman AM. Adenosine inhibits lipopolysaccharide-induced secretion of tumor necrosis factor-alpha in the failing human heart. Circulation 1998;97:521-524.

23. Keel M, Schregenberger N, Steckholzer U, Ungethum U, Kenney J, Trentz O, Ertel W. Endotoxin tolerance after severe injury and its regulatory mechanisms. J Trauma 1996;41:430-437.

4

24. Randow F, Syrbe U, Meisel C, Krausch D, Zuckermann H, Platzer C, Volk HD. Mechanism of endotoxin desensitization: involvement of interleukin 10 and transforming growth factor beta. J Exp Med 1995;181:1887-1892.

25. Garvy BA, Fraker PJ. Suppression of the antigenic response of murine bone marrow B cells by physiological concentrations of glucocorticoids. Immunology 1991;74:519-523.

26. Wilckens T. Glucocorticoids and immune function: physiological relevance and pathogenic potential of hormonal dysfunction. Trends Pharmacol Sci 1995;16:193-197.

27. Anker SD, Clark AL, Kemp M, Salsbury C, Teixeira MM, Hellewell PG, Coats AJS. Tumor necrosis factor and steroid metabolism in chronic heart failure: possible relation to muscle wasting. J Am Coll Cardiol 1997;30:997-1001.

28. Barnes PJ, Karin M. Nuclear factor-kappaB: a pivotal transcription factor in chronic inflammatory diseases. N Engl J Med 1997;336:1066-1071.

29. Lew WY, Ryan J, Yasuda S. Lipopolysaccharide induces cell shrinkage in rabbit ventricular cardiac myocytes. Am J Physiol 1997;272:H2989-H2993.

30. Bachetti T, Ferrari R. The dynamic balance between heart function and immune activation. Europ Heart J 1998;19:681-682.

31. Kelly RA, Smith TW. Cytokines and cardiac contractile function. Circulation 1997;95:778-781.

32. Torre-Amione G; Kapadia S; Lee J; Bies RD; Lebovitz R; Mann DL. Expression and functional significance of tumor necrosis factor receptors in human myocardium. Circulation 1995;92:1487-1493.

33. Tracey KJ, Morgello S, Koplin B, Fahey TJ III, Fox J, Aledo A, Manogue KR, Cerami A. Metabolic effects of cachectin/tumor necrosis factor are modified by site of production: Cachectin/tumor necrosis factor-secreting tumor in skeletal muscle induces chronic cachexia, while implantation in brain induces predominantely acute anorexia. J Clin Invest 1990;86:2014-2024.

34. Bristow MR. Tumor necrosis factor-α and cardiomyopathy. Circulation 1998;97:1340-1341.

35. Munger MA, Johson B, Amber IJ, Callahan KS, Gilbert EM. Circulating concentrations of proinflammatory cytokines in mild or moderate heart failure secondary to ischemic or ideopathic dilated cardiomyopathy. Am J Cardiol 1996;77:723-727.

36. Keith M, Geranmayegan A, Sole MJ, Kurian R, Robinson A, Omran AS, Jeejeebhoy KN. Increased Oxidative Stress in Patients With Congestive Heart Failure. J Am Coll Cardiol 1998;31:1352-1356.

37. Anker SD, Volterrani M, Egerer KR, Felton CV, Kox WJ, Poole-Wilson PA, Coats AJS. Tumor necrosis factor alpha as a predictor of peak leg blood flow in patients with chronic heart failure. Q J Med 1998;91:199-203.

38. Tracey KJ, Cerami A. Tumor necrosis factor, other cytokines and disease. Ann Rev Cell Biol 1994;10:317-43.

39. Yan SF, Tritto I, Pinsky D, Liao H, Huang J, Fuller G, Brett J, May L, Stern D. Induction of interleukin-6 (IL-6) by hypoxia in vascular cells. J Biol Chem 1995;270:11463-11471.

40. Munger MA, Stanek EJ, Nara AR, Strohl KP, Decker MJ, Nair RN. Arterial oxygen saturation in chronic congestive heart failure. Am J Cardiol 1994;73:180-185.

41. Klein CL, Kohler H, Bittinger F, Otto M, Hermanns I, Kirkpatrick CJ. Comperative studies on vascular endothelium in vitro. 2.Hypoxia: its influences on endothelial cell proliferation and expression of cell adhesion molecules. Pathobiology 1995;63:1-8.

42. Eggesbo JB, Hjermann I, Lund PK, Joo GB, Ovstebo R, Kierulf P. LPS-induced release of IL-1 beta, IL-6, IL-8, TNF-alpha and sCD14 in whole blood and PBMC from persons with high or low levels of HDL-lipoprotein. Cytokine 1994;6:521-529.

Example 2: Experimental trials relating to the use of compounds able to inhibit the response by a cell to endotoxin (LPS) in treating chronic heart failure or acute heart failure.

Invasive assessments looking for endotoxin levels in different locations in the body (left and right ventricle, hepatic vein, renal vein, peripheral vein and artery, coronary sinus) may be made in patients with decompensated CHF and myocardial infarction.

This may help in confirming the source of the endotoxin. If endotoxin is highest in the hepatic vein this may indicate that the liver or more likely the bowel is the source of endotoxin (LPS). Further, if endotoxin is higher in the hepatic vein compared to the left ventricle the lung may be excluded as a source of endotoxin.

Gut permeability assessments may be made using sugar absorption tests in patients with and without oedema and control subjects. The precise mechanism of endotoxin uptake through the bowel is not clear; sugar

absorption may reflect this pathway. However, kidney dysfunction (frequent in heart failure) may complicate interpretation of the results.

The relationship between endotoxin plasma levels and prognosis in oedematous and non-oedematous heart failure patients may be investigated.

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Table 1: Characteristics of chronic heart failure (CHF) patients with and without peripheral edema compared to healthy volunteers.

	healthy volunteers	CHF - no edema	CHF - edema	p (ANOVA)
n	14	20	20	
age	55 ± 4	63 ± 4	64 ± 2	
NYHA class		2.6 ± 0.2	3.3 ± 0.1 ###	
weight [kg]	74 ± 7	76 ± 7	78 ± 8	
etiology: ischemic		16	11	
idiopathic dilative		4	9	
sodium [mmol/L]	139 ± 0.4	137 ± 1.2	134 ± 1.1 **	< 0.006
creatinine [μmol/L]	82 ± 4	131 ± 14	219 ± 37 *** #	< 0.003
urea [mmol/L]	5.4 ± 0.2	11.0 ± 2.0	20.0 ± 2.9 *** ##	< 0.0003
uric acid [μmol/L]	308 ± 17	417 ± 42 *	640 ± 53 *** ###	< 0.0001
ASAT [IU/L]	26 ± 3	24 ± 2	23 ± 2	
ALAT [IU/L]	23 ± 3	17 ± 1 *	14 ± 1 ##	< 0.01

Legend: *: $p < 0.05$, **: $p<0.01$, ***: $p<0.001$ vs healthy volunteers; #: $p<0.05$, ##: $p<0.01$, ###: $p<0.001$ vs no edema; NYHA, New York Heart Association; ASAT, aspartate aminotransferase; ALAT, alanine aminotransferase

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Table 2: Plasma levels of endotoxin and inflammatory markers in healthy volunteers and patients with chronic heart failure (CHF).

	healthy volunteers	CHF - no edema	CHF - edema	p (ANOVA)
endotoxin [IU/mL]	0.46 ± 0.05	0.37 ± 0.05	0.74 ± 0.10 * ###	< 0.003
TNF-α [pg/mL]	24.6 ± 2.4	25.8 ± 1.8	36.6 ± 2.8 ** ##	< 0.001
sTNF-R1 [pg/mL]	708 ± 57	1077 ± 118	1922 ± 313 *** ###	< 0.001
sTNF-R2 [pg/mL]	1465 ± 264	2096 ± 330	3143 ± 388 ** #	< 0.01
sCD14 [ng/mL]	3456 ± 156	3674 ± 102	4243 ± 154 *** ###	< 0.001
procalcitonin [ng/ml]	87 ± 4	106 ± 16	145 ± 21	= 0.073
interleukin-6 [pg/mL]	2.0 ± 0.1	4.3 ± 1.2	14.7 ± 3.9 ** ###	< 0.003
CRP [mg/L]	5.6 ± 0.5	9.5 ± 1.6	19.7 ± 4.6 ** #	< 0.003

Legend: *: p< 0.05, **: p<0.01, ***: p<0.001 vs healthy volunteers; #: p<0.05, ##: p<0.01, ###: p<0.001 vs no edema;
TNF, tumor necrosis factor; sTNFR, soluble TNF receptor; sCD14, soluble CD14; CRP, c-reactive protein

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CLAIMS

1. A method of treating, preventing or ameliorating chronic heart failure or acute heart failure in a patient the method comprising administering to the patient an effective amount of a compound that is able to inhibit the response by a cell to endotoxin (lipopolysaccharide; LPS).

2. A method of treating, preventing or ameliorating endotoxin-mediated immune activation in acute or chronic heart failure in a patient the method comprising administering to the patient an effective amount of a compound that is able to inhibit the response by a cell to endotoxin (lipopolysaccharide; LPS).

3. A method according to Claim 1 or 2 wherein the compound is able to decrease the cytokine production by a cell in response to endotoxin (lipopolysaccharide; LPS).

4. A method according to any one of claims 1 to 3 wherein the compound is a corticosteroid.

5. A method according to any one of claims 1 to 4 wherein the compound is able to inhibit signalling *via* the CD14 receptor.

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6. A method according to any one of the preceding claims wherein the compound is administered orally.

~~7. A method according to any one of Claims 1 to 5 wherein the~~ compound is administered intravenously.

8. Use of a compound that is able to inhibit the response by a cell to endotoxin (lipopolysaccharide; LPS) in the manufacture of a medicament for treating, preventing or ameliorating chronic heart failure or acute heart failure in a patient.

9. Use of a compound that is able to inhibit the response by a cell to endotoxin (lipopolysaccharide; LPS) in the manufacture of a medicament for treating, preventing or ameliorating endotoxin-mediated immune activation in acute or chronic heart failure in a patient.

10. The use of claim 8 or claim 9 wherein the compound is a corticosteroid.

11. A method of treating, preventing or ameliorating chronic heart failure or acute heart failure in a patient the method comprising administering to

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the patient an effective amount of a corticosteriod or an antibody able to bind the CD14 receptor.

12. A method of treating, preventing or ameliorating endotoxin-mediated immune activation in acute or chronic heart failure in a patient the method comprising administering to the patient an effective amount of a corticosteriod or an antibody able to bind the CD14 receptor.

13. The method or use of any of the preceding claims wherein a diuretic is administered to the patient.

14. A pharmaceutical formulation comprising a compound that is able to inhibit the response by a cell to endotoxin (lipopolysaccharide; LPS) and a diuretic.

15. Any novel method of treating, preventing or ameliorating acute or chronic heart failure as herein disclosed.

16. Any novel pharmaceutical composition as herein disclosed.

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ABSTRACT

THERAPY AND USE OF COMPOUNDS IN THERAPY (4)

A method of treating, preventing or ameliorating chronic heart failure or acute heart failure in a patient the method comprising administering to the patient an effective amount of a compound that is able to inhibit the response by a cell to endotoxin (lipopolysaccharide; LPS).

A method of treating, preventing or ameliorating endotoxin-mediated immune activation in acute or chronic heart failure in a patient the method comprising administering to the patient an effective amount of a compound that is able to inhibit the response by a cell to endotoxin (lipopolysaccharide; LPS).

The compound may be able to decrease the cytokine production by a cell in response to endotoxin (lipopolysaccharide; LPS).

Figure 1

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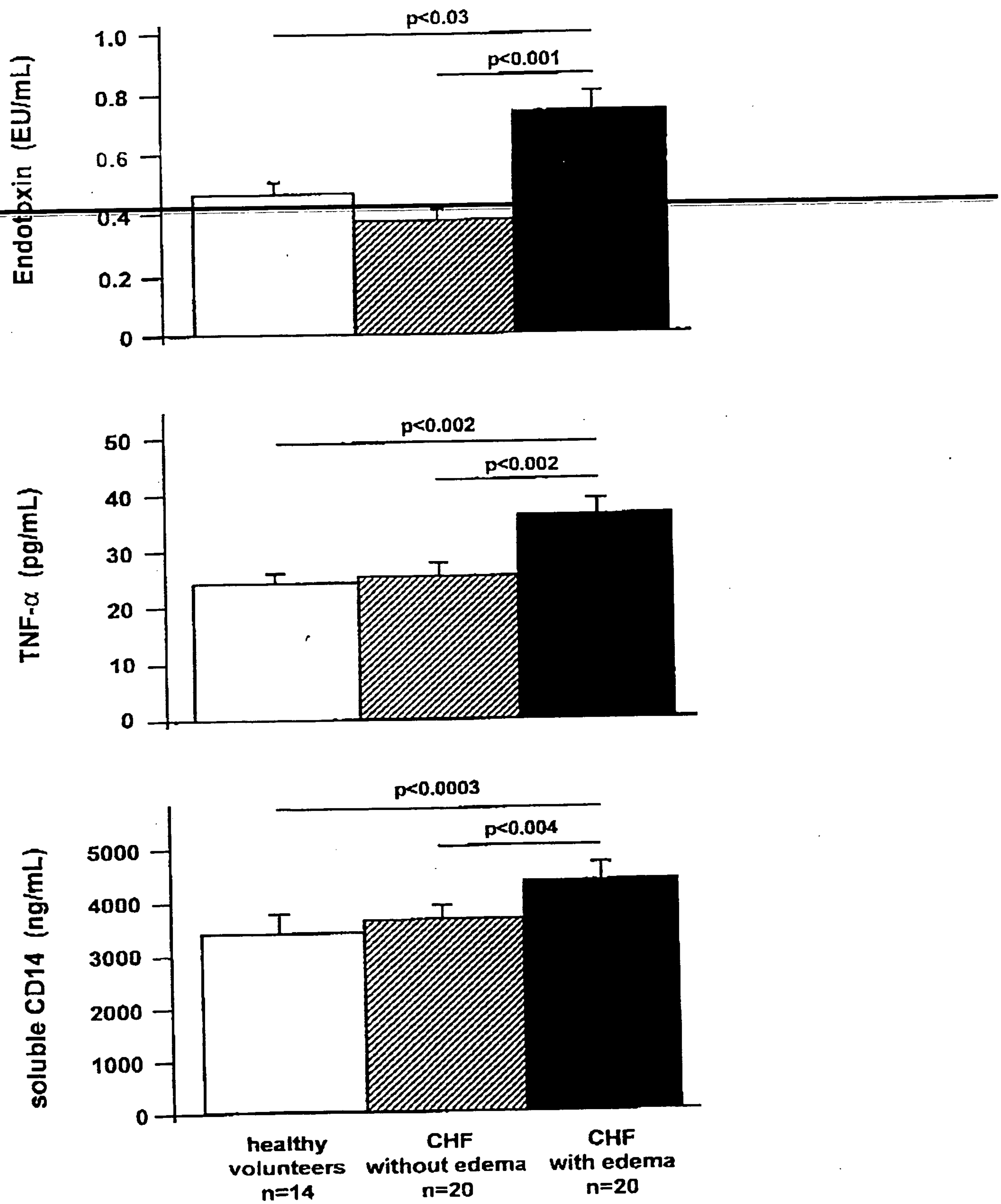
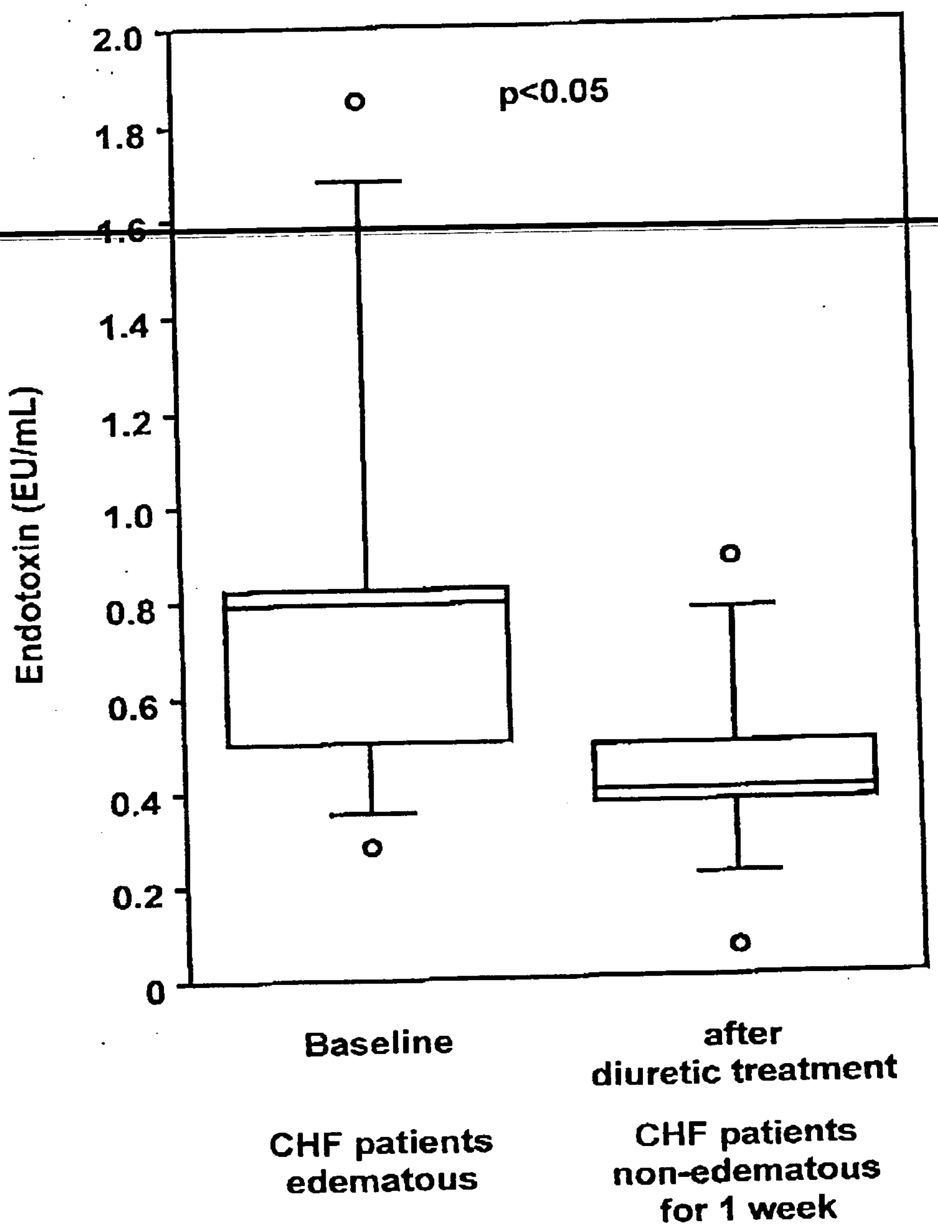
Figure 1


p<0.03
p<0.001
Endotoxin (EU/mL)
1.0
0.8
0.6
0.4
0.2
0
p<0.002
p<0.002
TNF-α (pg/mL)
50
40
30
20
10
0
p<0.0003
p<0.004
soluble CD14 (ng/mL)
5000
4000
3000
2000
1000
0
healthy volunteers n=14
CHF without edema n=20
CHF with edema n=20

4

2/2

Figure 2



p<0.05
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
Endotoxin (EU/mL)
Baseline
after diuretic treatment
CHF patients edematous
CHF patients non-edematous for 1 week